Use of 25-hydroxyvitamin D3 and dietary calcium to improve tenderness of beef from the round of beef cows

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Use of 25-hydroxyvitamin D_3 and dietary calcium to improve tenderness of beef from the round of beef cows[1,2]

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ABSTRACT: The objective of this trial was to determine how 25-hydroxyvitamin D_3 (25-OH D_3) supplementation, altering supplemental dietary calcium, or their combination influence postmortem biochemical and tenderness changes in muscles from the round of mature cows. Twenty-seven Angus cows (3 to 7 yr old) were allotted randomly to 9 pens with 3 cows per pen. Treatments were arranged in a 3 × 3 factorial design with 3 dosages of 25-OH D_3 (0, 250, or 500 mg of 25-OH D_3 administered as a 1-time oral bolus 7 d before slaughter) and 3 percentages of supplemental limestone (0.5, 0.75, and 1.0%) replenished in the diet for 3 d before slaughter and after a 2-wk limestone withdrawal. Plasma samples were obtained during the feeding period. Upon slaughter, adductor, gracilus, pectineus, sartorius, semimembranosus, vastus intermedius, and vastus lateralis muscles were obtained and aged for 1, 3, or 7 d. Calcium concentrations were increased in plasma when 250 or 500 mg of 25-OH D_3 were administered ($P \leq 0.05$). Calcium concentrations in muscle increased ($P \leq 0.001$) when 500 mg of 25-OH D_3 were administered. Concentrations of 25-OH D_3 in meat and in plasma and 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2$ D_3] in plasma were increased when 25-OH D_3 was administered ($P \leq 0.05$). The percentage of limestone replenished in the diet had no effect on 25-OH D_3 or 1,25-$(OH)_2$ D_3 in meat or in plasma. Calpastatin activity was affected by treatments only in the gracilus and vastus intermedius muscles ($P \leq 0.05$). Among all muscles and aging periods, calpastatin activity and intensity of troponin-T degradation product were related inversely. Results indicate that supplemental 25-OH D_3 has some influence on muscle characteristics known to improve tenderness, but improved tenderness was not observed.

Key words: beef, calcium, cow, 25-hydroxyvitamin D_3, tenderness

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INTRODUCTION

Beef from culled cows is generally less tender and, therefore, less desirable than that from younger cattle. As cattle age, collagen cross links increase and cause beef to become less tender, which is a factor that may confound attempts at premortem tenderization. Effectiveness of methods that are known to improve tenderness of beef from younger cattle has not been fully investigated in mature cows. Dietary vitamin D_3 improves tenderness of beef from younger cattle by accelerating the postmortem aging process (Swanek et al., 1999; Montgomery et al., 2000, 2002; Karges et al., 2001). The proposed mechanism by which vitamin D_3 improves tenderness is via the calpain protease system, which is responsible for postmortem proteolysis and subsequent tenderization of beef (Koohmaraie et al., 1991). One possible concern of administering large dosages of vitamin D_3 is that residues of vitamin D_3 activity in beef are high enough to cause hypervitaminosis D in humans if large quantities of beef were consumed on a regular basis (Swanek et al., 1999; Montgomery et al., 2000, 2002; Karges et al., 2001). The 25-hydroxyvitamin D_3 (**25-OH D_3**) has been investigated as a possible alternative to vitamin D_3 supplementation (Foote et al., 2004; Wertz et al., 2004). Results from the studies of Foote

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Table 1. Experimental design

Limestone, % of DM[1]	25-Hydroxyvitamin D_3, mg		
	0	250	500
0.50	3 cows[2]	3 cows	3 cows[2]
0.75	3 cows	3 cows	3 cows
1.00	3 cows[2]	3 cows	3 cows[2]

[1]Percentage of limestone that was replenished in the diet 3 d before slaughter.
[2]Indicates treatments used for determination of calpastatin activity.

et al. (2004) and Wertz et al. (2004) indicate that dietary 25-OH D_3 has the potential to favorably alter conditions within the muscle to support increased postmortem proteolysis and improved tenderness. Foote et al. (2004) and Wertz et al. (2004) did not observe improved beef tenderness as a result from treatment with 125 mg of 25-OH D_3, but the rather small dosage administered likely contributed to the null findings.

Additionally, research has indicated that plasma calcium concentration can be increased by withdrawing and then replenishing dietary calcium (Goings et al., 1974). The 25-OH D_3 has the potential to improve beef tenderness via the same mechanism as vitamin D_3 supplementation, and dietary calcium manipulations can elevate plasma calcium concentration. Some industry interest exists in finding more profitable routes for the sale of beef from culled cows.

Therefore, we hypothesized that 25-OH D_3 in conjunction with manipulations of dietary calcium would increase plasma calcium concentrations and improve tenderness of muscles from the round section of beef cows. Seven muscles were selected to test this hypothesis.

MATERIALS AND METHODS

Animals

Approval for this project was obtained from the Iowa State University Animal Care and Use Committee, and all regulations were followed.

Twenty-seven Angus cows (3 to 7 yr of age) were obtained from a breeding project at Iowa State University. The cows were housed at the Iowa State University Beef Nutrition Research Farm in Ames, IA. Cows were allotted by BW to 1 of 9 dietary treatments. The average age was similar for each treatment. Nine treatments arranged with 3 dosages of 25-OH D_3 (0, 250, or 500 mg of 25-OH D_3) and 3 manipulations of supplemental dietary calcium (limestone), which was withdrawn from all diets for 2 wk and then replenished at 0.5, 0.75, or 1.0% of diet DM (Table 1). The group of cows receiving 0 mg of 25-OH D_3 and 0.5% limestone upon replenishment was considered the control group. Cows were housed in 9 pens of 3 cows, and all cows in each pen were in the same dietary treatment. The 25-OH D_3 (Rovimix Hy-D 1.25) was obtained from DSM Nutritional Products Inc., Ames, IA. Dosages of 25-OH D_3 and cornstarch as the placebo (36.23 g; based on the weight of Rovomix Hy-D 1.25 to supply 500 mg of 25-OH D_3) were weighed and divided evenly into 5 gelatin capsules.

Experimental Design

The experimental timeline is shown in Figure 1. Cows had been on pasture since calving in the spring. On the day cows were brought to the feedlot, a blood sample was obtained, and then cattle were fed a high-concentrate diet (Table 2) for 28 d. Seventeen days before slaughter, supplemental calcium was withdrawn from

Table 2. Diet composition[1]

Ingredient	% of diet, DM basis	Ca, % in feed ingredient	Ca contribution to the diet, % in 0.50% limestone diet	Ca contribution to the diet, % in 0.75% limestone diet	Ca contribution to the diet, % in 1.0% limestone diet
Cracked corn	78.88	0.05	0.04	0.04	0.04
Corn silage	15.00	0.08	0.01	0.01	0.01
Soybean meal	2.64	0.28	0.01	0.01	0.01
Steep liquor	1.00	—	—	—	—
Cane molasses	0.91	0.74	0.01	0.01	0.01
Urea	0.64	0.09	0.001	0.001	0.001
Limestone, 37.1% Ca	0.50	37.1	0.19	0.28	0.37
Sodium chloride	0.30	—	—	—	—
Vitamin A premix[2]	0.08	—	—	—	—
Trace mineral premix	0.02	—	—	—	—
Rumensin[3]	0.02	—	—	—	—
Total	100	—	0.26%	0.35%	0.44%

[1]All cows were fed the diet containing 0.50% limestone until 17 d before slaughter. When supplemental calcium was replenished 3 d before slaughter, cows received the 0.50, 0.75, or 1.0% limestone diet. When limestone was supplemented at 0.75 or 1.0% of the diet, that amount was added to the total diet.
[2]Vitamin A premix provided 30,000 IU of vitamin A/100 kg of feed.
[3]Rumensin (Elanco, Indianapolis, IN) provided 0.0024% monensin in the diet.

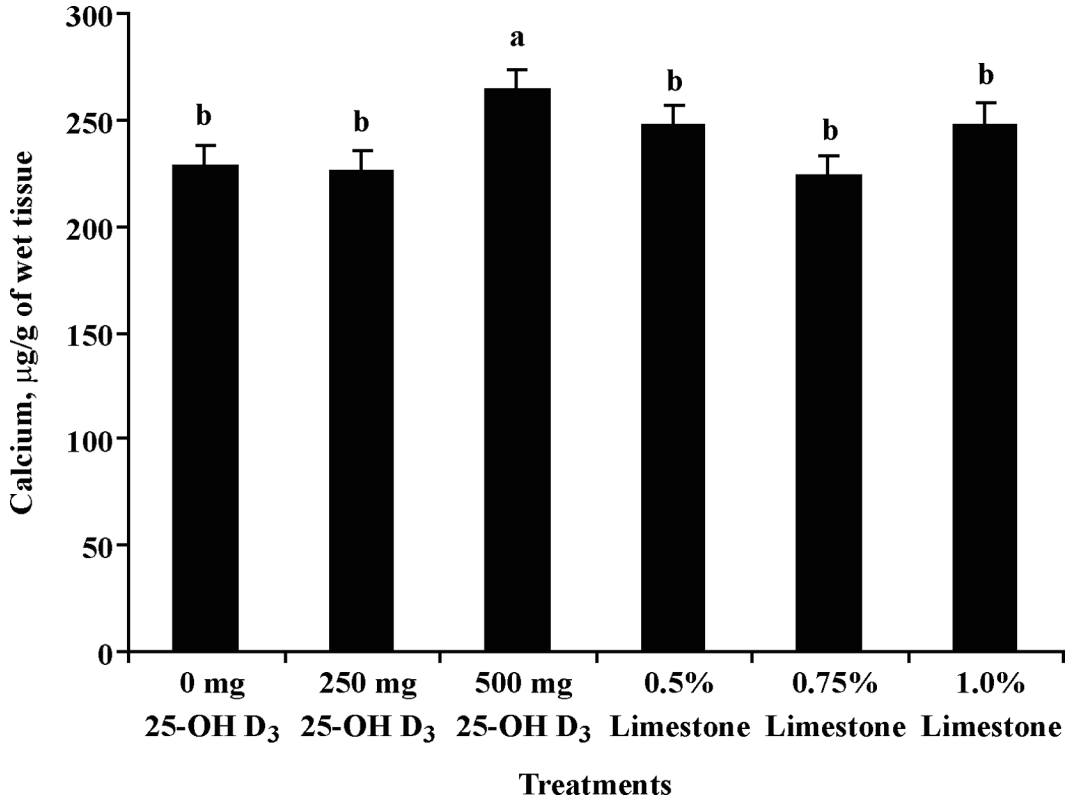

Figure 1. Calcium concentration in muscle of cows treated with 25-hydroxyvitamin D_3 (25-OH D_3) and manipulations of dietary calcium. No muscle differences existed, so data from all muscles were pooled. [a,b]Bars having the different letters differ; treatment with 500 mg of 25-OH D_3 increased calcium concentration in muscle compared with dosages of 0 or 250 mg of 25 OH D_3 and differing concentrations of replenished limestone ($P \leq 0.05$), whereas withdrawing and then replenishing different amounts of limestone in the diet did not affect muscle calcium concentration ($P > 0.05$).

the diet, and a blood sample was drawn. An additional blood sample was obtained 7 d after supplemental calcium was withdrawn (10 d before slaughter). Seven days before slaughter, another blood sample was obtained, and 25-OH D_3 (0, 250, or 500 mg of 25-OH D_3) was administered by oral bolus. Additional blood samples were obtained 5, 3, and 1 d before slaughter. Blood was collected by using 3.81-cm, 20-ga needles and sodium-heparinized Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ). All blood samples immediately were stored on ice until centrifugation. Plasma was stored at –20°C until analyses. Supplemental calcium was replenished in the diet (limestone at 0.5, 0.75, or 1.0% of diet DM) 3 d before slaughter (Table 2). Calcium concentration was adequate for maintenance for beef cattle according to the NRC (2000). That is, the calcium concentration was 0.26% of diet DM in the diet containing 0.5% limestone. The concentration was 0.35 and 0.44% in the 0.75 and 1.0% limestone diets, respectively.

Cows were transported 55 km to be slaughtered at Amend Meat Packing in Des Moines, IA, or were transported 1.6 km to be slaughtered at the Iowa State University Meat Laboratory, Ames, IA. To be consistent, regardless of location of slaughter, rounds were obtained 20 h after slaughter and processed at the Iowa State University Meat Laboratory, Ames, IA. Seven muscles (adductor, gracilus, pectineus, sartorius, semimembranosus, vastus intermedius, and vastus lateralis) were dissected by experienced personnel. Each muscle was sliced into six 2.54-cm steaks, and each steak was vacuum-packaged individually. Two steaks from each muscle from each cow immediately were frozen at –20°C. Those steaks were considered aged for 1 d. The remaining 4 steaks from each muscle from each cow were refrigerated at 4°C until 3 or 7 d of aging and then were frozen at –20°C. One steak from each aging period was used for biochemical analyses and the other for tenderness analysis. An additional small sample was obtained for immediate determination of calpastatin activity.

Calpastatin Activity in Beef

After dissection, a fresh piece from each muscle from each of the cows in 4 of the treatments (0 mg of 25-OH D_3, 0.5% limestone; 500 mg of 25-OH D_3, 0.5% limestone; 0 mg of 25-OH D_3, 1.0% limestone; and 500 mg of 25-OH D_3, 1.0% limestone; Table 1) was used immediately for calpastatin analysis (Lonergan et al., 2001; Maddock et al., 2005). These treatments were chosen because it was not feasible to test all of the samples for

calpastatin activity, and these treatments were those in which we expected the greatest treatment differences. Briefly, 10 g of each muscle (adductor, gracilus, pectineus, sartorius, semimembranosus, vastus intermedius, and vastus lateralis) from each cow were homogenized in 30 mL of postrigor extraction buffer (100 m*M* Tris, 10 m*M* EDTA, pH 8.3) with 100 mg/L of ovomucoid, 2 m*M* phenylmethylsulphonylfluoride, and 4 μ*M* *trans*-epoxysuccinyl-L-leucylamido(4-guanidino) butane L-*trans*-3-carboxyoxiran-2-carbonyl-L-leucylagmatine (E64, Sigma, St. Louis, MO) added just before homogenization. Samples were homogenized for 30 s in a polytron blender (Brinkmann Instruments, Westbury, NY). Samples were allowed to rest for 30 s; then, the cycle of homogenizing and resting was repeated twice so that the samples were homogenized 3 times. The homogenate of each sample was transferred equally to 2 centrifuge tubes, and the blender was rinsed with 10 mL of postrigor extraction buffer. The rinse was added to the centrifuge tubes. Samples were centrifuged for 30 min at 34,000 × *g* at 4°C. Protein content of the supernatant was determined (Bradford, 1976) by using premixed reagents (BioRad, Hercules, CA). The supernatant was dialyzed overnight against 40 m*M* Tris, pH 7.5, and 1 m*M* EDTA. The dialyzed supernatant was transferred to a conical centrifuge tube, heated in a 95°C water bath for 20 min, and then cooled in an ice bath for 15 min. Each sample was clarified by centrifugation for 30 min at 34,000 × *g* at 4°C. The supernatant was filtered through cheesecloth into a 50-mL conical centrifuge tube. The volume of the supernatant was recorded, and the samples were stored at 4°C until analysis. Calpastatin activity was measured by using a known amount of m-calpain activity (Koohmaraie et al., 1995). Samples were measured in duplicate.

Calcium and Magnesium Concentration in Plasma and Calcium Concentration in Beef

Briefly, 5 mL of 0.1% lanthanum oxide was added to 100 μL of plasma from each cow; the mixture was vortexed and analyzed by using atomic absorption spectroscopy (Perkin-Elmer Corp., Norwalk, CT). The same sample was used to measure the concentrations of calcium and magnesium in plasma. Meat samples were prepared for total calcium analysis by weighing 3 g of each muscle (aged 1 d) from each cow into an acid-washed beaker and then using a modified HNO_3-H_2SO_4 wet combustion method (NMAM, 1994). The resulting solution was standardized to 25 mL with distilled deionized water and then analyzed by using atomic absorption (Perkin-Elmer Corp.). All samples were analyzed in duplicate (AOAC, 1990).

25-Hydroxyvitamin D_3 and 1,25-Dihydroxyvitamin D_3 in Plasma

The 25-OH D_3 in plasma was extracted with acetonitrile and quantified in duplicate by RIA using ^{125}I-labeled 25-OH D_3 (Diasorin, Stillwater, MN) as the tracer (Hollis et al., 1993). Similarly, 1,25-dihydroxyvitamin D_3 [**1,25-$(OH)_2$ D_3**] was extracted by using acetonitrile, but that extract was treated with sodium periodate and purified by using solid phase extraction. The 1,25-$(OH)_2$ D_3 was quantified from the purified sample by RIA using ^{125}I as the tracer (Hollis et al., 1996). The intraassay CV was 7.5%, and the interassay CV was 14.7%. The detection limit was 2.4 ng/L.The standard used was 1,25-$(OH)_2$ D_3 (D1530) purchased from Sigma.

25-Hydroxyvitamin D_3 in Meat

The 25-OH D_3 concentrations in meat were determined in the semimembranosus for all cows and in adductor, gracilus, pectineus, sartorius, vastus intermedius, and vastus lateralis for cows given 500 mg of 25-OH D_3 and fed 1.0% limestone by using HPLC (Horst et al., 1981). Briefly, 3 g of meat were homogenized in 12 mL of phosphate-buffered saline (0.14 *M* NaCl and 0.01 *M* K_2HPO_4; pH 7.0). Samples were extracted with chloroform:methanol (2:1, vol:vol); then, the extracts were dried by using a Savant SpeedVac concentrator (Thermo Electron Corp., Milford, MA). The resulting residues were dissolved in 1 mL of hexane and applied to silica columns (Varian, Harbor City, CA) to separate the 25-OH D_3 from the 1,25-$(OH)_2$ D_3. Known amounts of 25-hydroxyvitamin D_2 (internal standard; catalog number 17937, Sigma) then were added to each tube containing the 25-OH D_3 fraction from each sample, and the solvents were removed in the SpeedVac. The dry samples were suspended in 150 μL of the HPLC mobile phase (hexane:isopropanol, 92:8, vol/vol). Samples were injected onto a Dupont Zorbax NH_2 4.6 × 250-mm HPLC column (Mac-Mod Analytical, Chads Ford, CA). The flow rate of the mobile phase was 2 mL/min. A calibration curve of 25-OH D_3 was used to determine concentration of 25-OH D_3 in samples. The 1,25-$(OH)_2$ D_3 was not determined in meat samples because other research has shown that its concentration in meat is not affected by treatment with 25-OH D_3 (Wertz et al., 2004).

SDS PAGE and Western Blotting

Meat samples aged 1, 3, or 7 d from each cow were prepared for quantification of the 30-kDa degradation product of troponin-T that results from postmortem proteolysis (Huff-Lonergan et al., 1996a). Samples were prepared for gel electrophoresis by homogenizing 1 g of muscle in 12 mL of PBS. The homogenate was centrifuged for 20 min at 1,500 × *g* at 4°C. The supernatant was removed and analyzed for protein content by using prepared reagents for the Folin Lowry method (BioRad, Hercules, CA). Protein concentration of each sample was adjusted to 6.14 mg/mL with PBS. Sodium dodecylsulfate and β-mercaptoethanol were added, and samples were incubated at 60°C for 20 min. Samples were

Table 3. Effect of treatment with 25-hydroxyvitamin D_3 (25-OH D_3) and dietary calcium on calpastatin activity in beef muscle

		Calpastatin activity,[1] U/g of protein extracted						
25-OH D_3, mg	Limestone, % of DM	Gracilus	Adductor	Pectineus	Semimembranosus	Sartorius	Vastus lateralis	Vastus intermedius
0	0.5	83[b]	45	54	46	40	62	96[b]
0	1.0	41[a]	33	49	32	37	47	45[a]
500	0.5	45[a]	37	43	44	41	56	83[b]
500	1.0	59[ab]	32	46	42	44	93	63[a]
	SEM	7.0	4.7	4.8	8.0	4.0	16	12

[a,b]Means in the same column that do not have a common superscript letter differ, $P < 0.05$.
[1]1 unit of calpastatin activity is the amount of calpastatin required to inhibit 1 unit of calpain activity (Lonergan et al., 2001).

stored at –80°C until analysis. Protein degradation products were separated by PAGE. Every gel was loaded with samples, a reference standard for developing band density ratios, and a molecular weight marker. The reference sample used for determining extent of troponin-T degradation was a sample that had been prepared from the LM from a feedlot heifer and aged for 14 d. After the tracking dye reached the end of the gel, the proteins were transferred to a membrane for Western blotting (Huff-Lonergan et al., 1996b). The 30-kDa degradation product was detected by using antibody JLT-12 (Sigma Aldrich, St. Louis, MO) diluted 1:5,000 (vol/vol with PBS) as the primary antibody and antibody A-2554 (Sigma Aldrich) diluted 1:3,333 (vol/vol with PBS) as the secondary antibody. The ECL-Plus chemiluminescent system (Amersham Biosciences, GE Healthcare, Piscataway, NJ) was used to detect troponin-T. Membranes were visualized by using a 16-bit megapixel charge-coupled device camera (FlourChem8800, Alpha Innotech Corp., San Leandro, CA) and FluorChem IS-800 software (Version 3, Alpha Innotech Corp.). The band density ratios of the 30-kD degradation product of troponin-T in each sample relative to the reference standard were used to determine the extent of troponin-T degradation, and therefore the extent of postmortem proteolysis relative to samples from other treatments in this experiment.

Texture Analysis

Steaks were thawed for 24 h at 4°C. An industrial broiler (model CNO2, General Electric, Chicago Heights, IL) was preheated to medium high heat, and the adjustable grill grate was set 11.2 cm below the heat source. Steaks were broiled to an internal temperature of 35°C, turned, and broiled until the internal temperature reached 71°C (Pyrex digital probe oven thermometer, Cooks Emporium, Ames, IA). Each tray of broiled steaks was wrapped in plastic wrap (Saran Wrap, SC Johnson, Racine, WI), and all steaks were stored overnight at 4°C. The following morning, steaks were allowed to warm to ambient temperature (approximately 22°C). A texture analyzer (model TA-XTi, Texture Technologies Corp., Scarsdale, NY) fitted with a star probe blade was used to puncture steaks parallel to the muscle fibers at a penetration speed of 3.3 mm/s 3 times per steak. We chose to use the star probe instead of Warner-Bratzler shear force because 2 of the muscles, sartorius and pectineus, were not large enough to get 6 cores from all of the cows. By using the star probe on all of the samples, the data can be compared. The maximal force necessary to penetrate the steak was recorded, and the 3 values for each steak were averaged for statistical analysis. The coefficient of variance within each steak ranged from 2 to 12%, so 3 measurements per steak were considered sufficient.

Statistical Analysis

Treatments were arranged as a 3×3 factorial design with 3 dosages of 25-OH D_3 (0, 250, or 500 mg) and 3 concentrations of dietary calcium replenished in the diet (limestone at 0.5, 0.75, or 1.0% of diet DM). Each combination was analyzed as a separate treatment, and the MIXED procedure (SAS Inst. Inc., Cary, NC) was used to conduct ANOVA. Cow was the experimental unit. Star probe shear force and troponin-T degradation were analyzed as a repeated measure with aging day as the repeated variable. The age of the cows was used as a covariate to correct for the wide range of ages (3 to 7 yr). Least squares means were computed for all fixed effects and separated by using pairwise *t*-tests (PDIFF) when the *F*-test was significant ($P \leq 0.05$ unless otherwise noted) was detected.

RESULTS AND DISCUSSION

Calpastatin Activity in Muscle

Calpastatin is the inhibitor of calpain, which is responsible for postmortem proteolysis, so its inhibitory activity is an indication of beef tenderness. Calpastatin activities for each muscle are shown in Table 3. One unit of calpastatin activity is defined as the amount of calpastatin necessary to inhibit 1 unit of calpain activity. Calpastatin activity was lower in the gracilus muscle when 0 mg of 25-OH D_3 was administered with 1.0% limestone (41 U/g of protein) and when 500 mg of 25-OH D_3 was administered with 0.5% limestone (45 U/g of protein) as compared with activity in the gracilus

Table 4. Plasma calcium concentrations (mg/dL) of cows treated with 25-hydroxyvitamin D_3 (25-OH D_3) and manipulations of dietary calcium

25-OH D_3, mg	Limestone, % of DM	Time before slaughter, d						
		45	17	10	7	5	3	1
0	0.50	10.0	9.9	9.6	8.4	9.1	9.2[ac]	10.1[a]
0	0.75	9.3	9.3	9.1	7.9	8.8	8.2[a]	8.7[a]
0	1.00	8.0	8.1	8.2	7.8	8.6	8.8[ac]	9.8[a]
250	0.50	8.5	9.0	8.6	8.6	9.3	9.2[ac]	11.6[bc]
250	0.75	9.7	9.9	9.4	8.8	9.1	9.8[bc]	11.5[bc]
250	1.00	9.1	9.2	9.4	8.7	9.5	9.9[bc]	10.9[b]
500	0.50	8.7	9.9	9.1	8.9	9.4	9.4[ac]	11.5[bc]
500	0.75	8.0	9.2	8.5	8.0	9.7	9.3[ac]	11.9[c]
500	1.00	9.6	9.8	9.3	8.3	9.4	10.7[b]	11.2[bc]
	SEM	0.48	0.3	0.26	0.46	0.24	0.41	0.32

[a–c]Means in the same column that do not have a common superscript letter differ, $P < 0.05$.

muscle obtained from animals in the control group ($P \leq 0.05$). Calpastatin activity was intermediate to the activities in the control group (83 U/g of protein), and the groups previously listed when 500 mg of 25-OH D_3 was administered and limestone was replenished at 1.0% of diet DM (59 U/g of protein). In the vastus intermedius muscle, calpastatin activity was higher in the control group (96 U/g of protein) compared with cows receiving 500 mg of 25-OH D_3 with limestone replenished at 0.5% of DM (45 U/g of protein). When calpastatin activity is higher, calpain inhibition is increased and less proteolysis should occur, so meat may be less tender (Morgan et al., 1993; Geesink and Koohmaraie, 1999).

Though not statistically significant, calpastatin activity in the adductor and pectineus muscles follows the expected pattern of activity with greater activity in the control group than in all other treatments. In a recent study, expression of calpastatin mRNA was decreased greatly in LM of culled Korean cows that were supplemented with 25-OH D_3 one time 6 d before slaughter (Cho et al., 2006). In that study, even though calpastatin mRNA was decreased by 85%, there was no difference in the amount of calpastatin protein present or in the Warner-Bratzler shear force. Cho et al. (2006) did not measure calpastatin activity, so whether or not the calpastatin present was active remains unclear. In sum, treatment with 25-OH D_3 and differing concentrations of dietary calcium had small effects on calpastatin activity in most muscles. However, in gracilus and vastus intermedius, calpastatin activity was decreased with supplemental 25-OH D_3. The difference in effect could be related to the dominant fiber type or to the relative amount of collagen in the muscles. Unfortunately, those analyses were not planned for this experiment.

Calcium Concentrations in Plasma and in Meat

Calcium concentrations in plasma of cows were not different among treatment groups until 3 d before slaughter (Table 4). When supplemental calcium was removed from the diets, concentration of calcium in the plasma decreased slightly in all cases; cows, however, were able to maintain their plasma calcium concentration at slightly lower physiologic concentrations presumably by resorbing bone minerals. Plasma magnesium concentration did not change when plasma calcium concentrations were maintained in the absence of supplemental dietary calcium but decreased when dietary calcium was sufficient (data not shown). That decrease in plasma magnesium concentration provides evidence that the increase in plasma calcium that is observed after supplementation with 25-OH D_3 results from increased absorption of dietary calcium and not mobilization from bone (Shils et al., 1998). On the day that calcium was replenished in the diet (3 d after 25-OH D_3 was administered), concentrations of plasma calcium before feeding were increased, which indicates that cows were able to increase plasma calcium concentrations in response to dietary 25-OH D_3. After calcium was replenished in the diet, plasma calcium concentrations were increased to a greater extent in cattle that were supplemented with 25-OH D_3 ($P \leq 0.05$). Whether cows were supplemented with 250 or 500 mg of 25-OH D_3 did not seem to make a difference as to the magnitude of increase in plasma calcium concentration ($P \geq 0.05$).

The calcium concentrations in plasma at the start of the study and 1 d before slaughter in groups that were treated with a one-time bolus of 250 or 500 mg of 25-OH D_3 7 d before slaughter were similar to the plasma calcium concentrations observed when 5×10^6 or 7.5×10^6 IU of vitamin D_3 are administered for 9 consecutive days (Montgomery et al., 2000) and when 6×10^6 IU of vitamin D_3 are administered for 4 or 6 d (Karges et al., 2001). The plasma calcium concentrations observed in this study are similar to values obtained by Carnagey et al. (2008), Karges et al. (2001), and Montgomery et al. (2000) when supranatural dosages of vitamin D_3 were administered for several days. Other researchers have not successfully increased plasma calcium concentrations with supplemental 25-OH D_3 (Foote et al., 2004; Wertz et al., 2004), but the failure to induce hy-

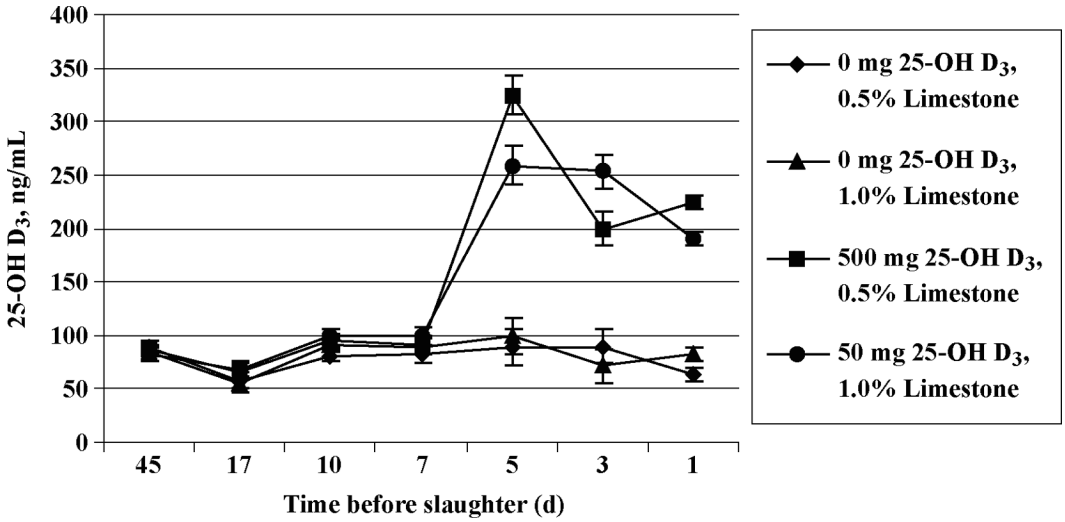

Figure 2. The 25-hydroxyvitamin D_3 (25-OH D_3) concentration in plasma of cows treated with 25-OH D_3 and dietary manipulations of calcium. Nonoverlapping error bars indicate that concentrations of 25-OH D_3 are greater at 5, 3, and 1 d before slaughter for cows treated with 500 mg of 25-OH D_3 than for those receiving no 25-OH D_3 with 0.5 or 1.0% of diet DM as limestone ($P \leq 0.05$). As expected, no differences in 25-OH D_3 concentration were observed from 45 to 7 d before slaughter ($P > 0.10$).

percalcemia in these previous studies probably resulted because of the lower dosage of 25-OH D_3 (125 mg) that was administered.

Total calcium concentration was measured in each muscle. Because no differences in calcium concentrations existed between muscles ($P \geq 0.20$), data for all muscles were combined into 1 data set and the main effect of supplemental 25-OH D_3 was analyzed. Total calcium concentration in muscles increased by approximately 12% when 500 mg of 25-OH D_3 was administered to cows as a single bolus 7 d before slaughter ($P \leq 0.05$; Figure 1). Unexpectedly, when limestone was replenished at 0.75% of diet DM, muscle calcium concentrations were lower than when limestone was replenished at 0.5 or 1.0% of diet DM ($P \leq 0.05$), but were similar to calcium concentrations in muscle of control cows ($P > 0.20$). The greatest increase in muscle calcium occurred when 500 mg of 25-OH D_3 was administered in conjunction with 1% limestone, but the interaction between 25-OH D_3 and limestone was not significant. The muscle calcium concentrations observed in the current study agree with observations from a study involving the use of 25-OH D_3 and vitamin E to improve tenderness (Carnagey et al., 2008). As with plasma calcium concentrations, experiments by Foote et al. (2004) did not elicit an increase in muscle calcium in response to treatment with 25-OH D_3. Again, this effect is probably attributable to the smaller dosage (125 mg) of 25-OH D_3 administered. Wertz et al. (2004) did not find an effect of treatment with 125 mg of 25-OH D_3 on water-soluble calcium concentration in LM, but they did not analyze the samples for total calcium concentration. The possibility exists that the increase in muscle calcium in the present study is attributable to the water insoluble calcium concentration in the muscle, but the likelihood of that possibility is small because Foote et al. (2004) used the same dosage of 25-OH D_3 as did Wertz et al. (2004), and they did not find an effect of treatment on total muscle calcium concentrations. In the present study, we found that increased plasma calcium concentrations were somewhat correlated with increased muscle calcium concentrations ($R^2 = 0.18$, $P < 0.001$), which supports the theory that the 125-mg dosage of 25-OH D_3 that was used by Foote et al. (2004) and by Wertz et al. (2004) was too low to elicit changes in muscle calcium concentrations.

25-OH D_3 and 1,25-$(OH)_2$ D_3 in Plasma

In plasma, 25-OH D_3 concentrations were increased from approximately 75 ng/mL to approximately 200 ng/

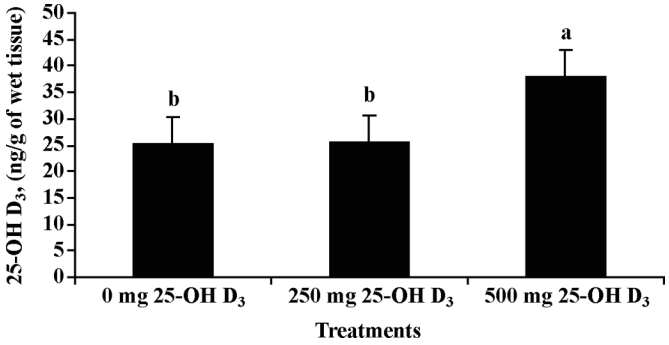

Figure 3. The 25-hydroxyvitamin D_3 (25-OH D_3) concentration in the semimembranosus muscle of cows treated with 25-OH D_3. [a,b]Means not bearing the same superscript differ ($P \leq 0.05$).

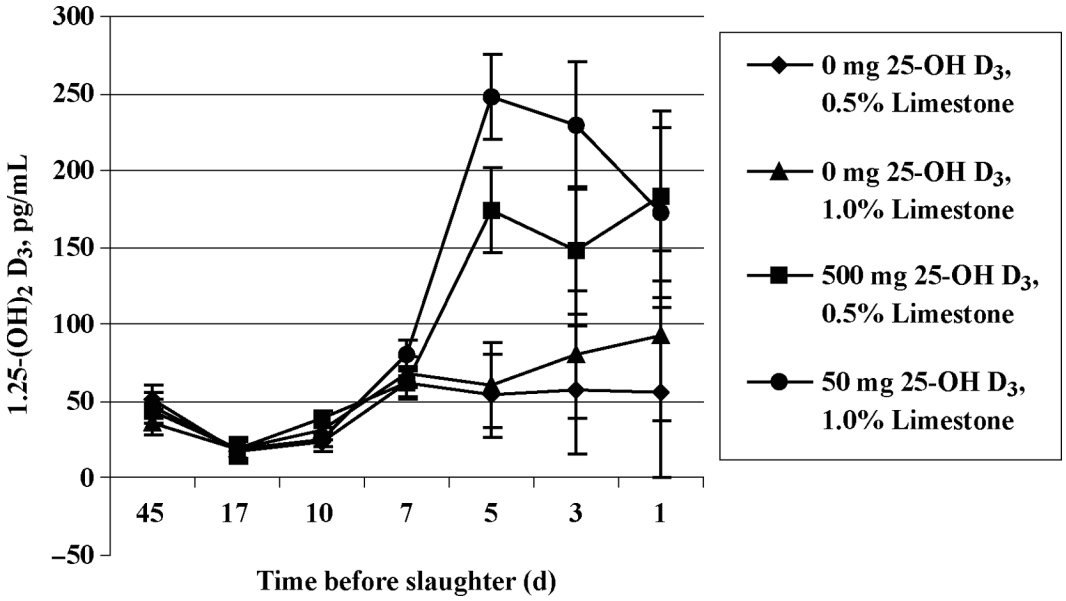

Figure 4. Plasma 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2$ D_3] concentration of cows treated with 25-hydroxyvitamin D_3 (25-OH D_3) and dietary manipulations of calcium. Nonoverlapping error bars indicates that concentrations of 1,25-$(OH)_2$ D_3 in plasma are higher at 5, 3, and 1 d before slaughter in cows treated with 500 mg of 25-OH D_3 than those receiving no 25-OH D_3 with 0.5% or 1.0% of diet DM as limestone ($P \leq 0.05$). As expected, no differences in 1,25-$(OH)_2$ D_3 concentration were observed from 47 to 7 d before slaughter ($P > 0.10$).

mL when cows were supplemented with either 250 or 500 mg of 25-OH D_3 ($P \leq 0.05$; Figure 2). The magnitude of increase in concentration of 25-OH D_3 in plasma is only about half of that observed in younger cattle (Wertz et al., 2004). For example, in a study conducted to determine the effects of feeding 25-OH D_3 and vitamin E to improve tenderness, concentrations of 25-OH D_3 in plasma were 13-fold higher in cows treated with 25-OH D_3 than in control animals (Carnagey et al., 2008). In the present study, the fold increase by the day before slaughter was approximately 2.5. Interestingly, the 25-OH D_3 concentration in control cows in this study was only slightly higher than that in a study conducted by Carnagey et al. (2008) in which younger heifers were used (80 vs. 60 ng/mL, respectively). The difference in baseline plasma 25-OH D_3 concentrations between these 2 studies probably can be attributed to the fact that the cows were housed outside on pasture before the start of this study (Hidiroglou et al., 1979). The concentrations of 25-OH D_3 observed in the current study are similar to those concentrations observed by Wertz et al. (2004) when 62.5 or 125 mg of 25-OH D_3 was administered 21, 7, 4, or 0 d before slaughter.

The 1,25-$(OH)_2$ D_3 concentrations in plasma from cows treated with 25-OH D_3 (250 or 500 mg) were 2.5 to 3 times higher than the concentrations observed in groups that were not supplemented with 25-OH D_3 ($P \leq 0.05$; Table 5). As expected, replenishing calcium in the diet did not affect the concentration of 1,25-$(OH)_2$ D_3 in plasma of cows ($P \geq 0.05$). Withdrawing supplemental calcium from the diet increased concentrations of 1,25-$(OH)_2$ D_3 in all cows on the day that 25-OH D_3 was administered. Blood samples were obtained before feeding, so the increase in 1,25-$(OH)_2$ D_3 concentration observed on the day that 25-OH D_3 was administered is a result of internal homeostatic mechanisms that are responsible for maintaining plasma calcium concentrations. The most likely mechanism is that low plasma calcium stimulates the parathyroid gland to produce more parathyroid hormone, which increases 1α-hydroxylase activity in the kidney and thereby increases 1,25-$(OH)_2$ D_3 concentrations in the plasma (Shils et al., 1998). The 1,25-$(OH)_2$ D_3 binds to nuclear receptors to increase transcription of calcium-binding protein in the intestine, which, in turn, increases efficiency of absorption of calcium from the diet and maintains plasma calcium concentrations. Overall, the concentrations in plasma of 1,25-$(OH)_2$ D_3 observed in the present study are similar to those concentrations observed by Montgomery et al. (2000) when 5 or 7.5×10^6 IU of vitamin D_3 was provided and those observed by Wertz et al. (2004) when 125 mg of 25-OH D_3 was provided, but lower than the concentrations observed by Foote et al. (2004) when 125 mg of 25-OH D_3 was administered one time 7 d before slaughter. The slightly higher concentration of 1,25-$(OH)_2$ D_3 at baseline is likely explained by the fact that cows were exposed to sunlight daily until the start of the study (Hidiroglou et al., 1979).

25-OH D_3 Concentration in Meat

The main effect of supplemental 25-OH D_3 was analyzed in samples from adductor, gracilus, pectineus, sartorius, semimembranosus, vastus intermedius, and

Table 5. Plasma 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2$ D_3] concentrations (pg/mL) of cows treated with 25-hydroxyvitamin D_3 (25-OH D_3) and differing amounts of dietary calcium

25-OH D_3, mg	Limestone, % of diet DM	Time before slaughter, d						
		45	17	10	7	5	3	1
0	0.50	52	17	23	61	54[a]	58[a]	55[a]
0	0.75	43	16	26	70	75[a]	76[a]	96[ab]
0	1.00	37	19	31	70	60[a]	81[a]	93[ab]
250	0.50	63	35	31	85	204[b]	185[b]	207[b]
250	0.75	44	28	36	73	195[b]	233[c]	162[b]
250	1.00	52	30	35	78	203[b]	201[b]	176[b]
500	0.50	44	20	39	62	174[b]	148[ab]	183[b]
500	0.75	49	39	31	74	218[b]	187[bc]	339[c]
500	1.00	47	20	25	80	248[b]	230[c]	172[b]
	SEM	8.2	6.5	5.2	11	28	42	55

[a–c]Means in the same column that do not have a common superscript letter differ, $P < 0.05$.

vastus lateralis muscles. No differences in 25-OH D_3 concentration were observed between muscles ($P \geq 0.10$; data not shown); thus, the semimembranosus is shown as an example of 25-OH D_3 concentration in muscle (Figure 3). The concentration of 25-OH D_3 in semimembranosus muscle was increased from 25 to 37 ng/g of fresh tissue with supplementation of 500 mg of 25-OH D_3 7 d before slaughter compared with concentrations in cows treated with 0 or 250 mg of 25-OH D_3 ($P \leq 0.05$; Figure 4). The concentrations of 25-OH D_3 observed in the present study are much higher than those concentrations observed by other researchers. One possible explanation for this finding is that the cows for this study were on pasture in the sun from the time of calving until August when cows were brought to the feedlot. Because UV radiation from the sun converts 7-dehydrocholesterol in the skin to vitamin D_3, it is likely that the vitamin D status of these cattle is much higher than the status of the cattle that were housed in a sheltered feedlot during fall and winter months because the body tissues can act as a reservoir for vitamin D_3 until it is metabolized (Hidiroglou et al., 1979).

Although statistically significant ($P < 0.05$), the concentration of 25-OH D_3 in steaks from cows supplemented with 500 mg of 25-OH D_3 is less than 2 times the concentration in the control samples (Figure 4). This concentration of 25-OH D_3 in beef is safe to consume even though it is higher than those concentrations observed in other studies. The current upper limit of vitamin D consumption is 50 μg of vitamin D_3/d (Standing Committee on Scientific Evaluation of Dietary Reference Intakes, 1997). The 25-hydroxyvitamin D_3 has about 1.4 times the activity of vitamin D on a weight basis, so, at the concentrations of 25-OH D_3 found in this study, a person would have to consume more than 567.5 g of meat daily to reach the upper limit for intake of vitamin D_3 activity. Because most people do not consume that much beef in a day, or at least on several consecutive days, this concentration is still reasonable. In fact, consuming beef from cattle supplemented with 25-OH D_3 is safe and may be a reasonable method to help ensure that consumers meet their daily recommended intake of vitamin D_3 activity (equivalent to 5 to 10 μg of vitamin D_3/d depending upon age; Standing Committee on Scientific Evaluation of Dietary Reference Intakes, 1997). A 170-g serving of beef from cows treated with 500 mg of 25-OH D_3 would provide 7 μg equivalent of vitamin D activity and meet the daily recommended intake of vitamin D_3 for most people without any harmful effects. New research indicates that the actual vitamin D requirement for healthy people might be as high as 2,200 IU per day (Heaney, 2005).

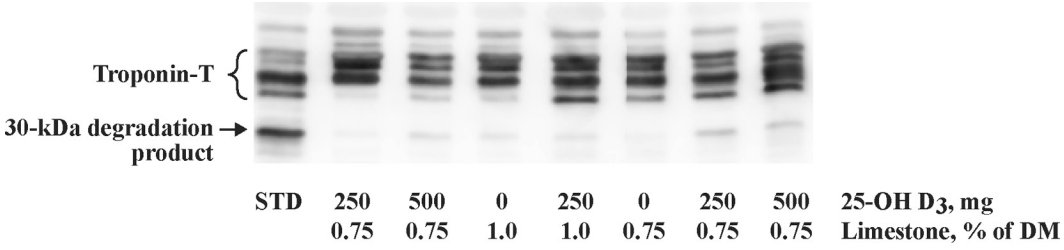

Figure 5. Postmortem protein degradation in samples from cows that were treated with 25-hydroxyvitamin D_3 (25-OH D_3) 7 d before slaughter. This figure shows degradation in the sartorius muscle after 3 d of aging. When 0.75% limestone was fed, degradation tended to be greater ($P < 0.10$) when 500 mg of 25-OH D_3 was supplemented than when 250 mg of 25-OH D_3 was supplemented. STD = standard.

Table 6. Effect of treatment with 25-hydroxyvitamin D_3 (25-OH D_3) and dietary calcium on troponin-T degradation in beef muscle[1]

Muscle and aging in days	0.5% limestone		1.0% limestone		SEM
	No 25-OH D_3	500 mg of 25-OH D_3	No 25-OH D_3	500 mg of 25-OH D_3	
Adductor					
1 d	$0.95^{b,z}$	0.23^{a}	0.22^{a}	$0.58^{a,y}$	0.14
3 d	$0.35^{b,y}$	0.30^{b}	0.19^{bc}	$0.02^{a,y}$	0.12
7 d	$0.24^{a,y}$	0.55^{a}	0.34^{a}	$2.65^{b,z}$	0.38
SEM	0.22	0.24	0.1	0.58	
Gracilus					
1 d	0.01^{y}	0.02^{y}	0.16	0.01	0.14
3 d	$0.02^{a,y}$	$0.03^{a,y}$	0.64^{b}	0.07^{a}	0.19
7 d	$0.16^{a,z}$	$0.23^{bc,z}$	0.63^{b}	0.09^{a}	0.17
SEM	0.05	0.07	0.32	0.06	
Pectineus					
1 d	0.15	0.07^{y}	0.16	0.32	0.19
3 d	1.03^{b}	$0.43^{a,y}$	0.64^{a}	0.39^{a}	0.41
7 d	0.18^{a}	$1.03^{b,z}$	0.66^{ab}	0.17^{a}	0.21
SEM	0.53	0.35	0.25	0.13	
Sartorius					
1 d	0.15	0.21	0.57	0.11	0.37
3 d	0.57	0.18	0.08	0.06	0.17
7 d	0.11	0.39	0.42	0.46	0.27
SEM	0.28	0.15	0.35	0.2	
Semimembranosus					
1 d	$0.01^{a,y}$	0.02^{a}	$0.17^{ab,y}$	0.56^{b}	0.18
3 d	$0.33^{ab,yz}$	0.50^{ab}	$0.05^{a,y}$	0.51^{b}	0.19
7 d	$0.67^{a,z}$	0.64^{a}	$1.38^{b,z}$	0.66^{a}	0.27
SEM	0.2	0.24	0.33	0.33	
Vastus intermedius					
1 d	0.02	0.07	0.27	0.03	0.11
3 d	0.09^{a}	0.08^{a}	0.08^{a}	0.94^{b}	0.26
7 d	0.08^{a}	0.10^{a}	0.59^{b}	0.14^{ab}	0.19
SEM	0.04	0.05	0.32	0.43	
Vastus lateralis					
1 d	0.03	0.02	0.21	0.01	0.16
3 d	0.17	0.52	0.12	0.22	0.17
7 d	0.08^{a}	0.32^{b}	0.17^{ab}	0.12^{ab}	0.1
SEM	0.07	0.22	0.12	0.09	

[a–c]Means in the same row that do not have a common superscript differ, $P < 0.10$.
[y,z]Means for same muscle within a column that do not have a common superscript differ, $P < 0.10$.
[1]Values represent band intensity as measured by densitometry in relation to a standard beef sample chosen from another study.

SDS PAGE and Western Blotting

The extent of troponin-T degradation was determined by SDS PAGE followed by Western blotting. Results are shown in Table 6, and an example is shown in Figure 5. Table 6 shows results from only 4 treatments for ease of viewing; data not presented support the conclusions associated with the results for treatments that are shown. Among all muscles and aging periods, calpastatin activity is correlated significantly with troponin-T degradation ($R^2 = -0.14$, $P = 0.02$), which shows, as expected, that higher calpastatin values correspond to decreased troponin-T degradation.

The extent of troponin-T degradation in the adductor at 7 d of aging was increased numerically with aging in all treatments and was increased significantly with treatment with 500 mg of 25-OH D_3. When 25-OH D_3 was used in combination with increased dietary calcium, the extent of protein degradation was increased ($P = 0.03$). In gracilus, in most cases the extent of troponin-T degradation increased with aging time and was increased to a greater extent in the 0 mg of 25-OH D_3, 1.0% limestone and 500 mg of 25-OH D_3, 0.5% limestone groups. These groups had the lowest 24-h calpastatin activities in gracilus muscle. Degradation was increased numerically with aging in the sartorius muscle but was increased to a greater extent with treatment with 500 mg of 25-OH D_3 or 1% limestone. In the semimembranosus muscle, aging increased troponin-T degradation numerically in all treatments but increased degradation significantly ($P = 0.042$) when limestone was fed at 1% of diet DM. In the vastus intermedius muscle at 7 d aging, more troponin-T degradation had occurred with 0 mg of 25-OH D_3, 1.0% limestone than in other treatments. Little troponin-T degradation was observed in the vastus lateralis regardless of treatment or aging period. The greatest extent of troponin-T degradation occurred when 500 mg of 25-OH D_3 was admin-

Table 7. Effect of 25-hydroxyvitamin D_3 (25-OH D_3) and manipulations of dietary calcium on star probe shear force[1] in selected muscles from the round of beef cows

	Adductor aging, d			Gracilus aging, d			Pectineus aging, d			Vastus intermedius aging, d		
Item	1	3	7	1	3	7	1	3	7	1	3	7
25-OH D_3, mg												
0	5.48[1]	6.02	5.62	4.04	3.49	4.34	4.38	4.47	4.03	5.19	5.03	5.28
250	5.50	5.55	5.42	3.85	3.60	3.80	5.02	5.08	4.65	5.32	5.57	4.66
500	5.83	5.61	5.76	4.17	4.00	4.59	4.72	4.89	4.46	5.51	5.47	5.12
SEM	0.24	0.40	0.52	0.34	0.30	0.45	0.19	0.34	0.28	0.21	0.32	0.38
Limestone, % of DM												
0.50	5.89	6.30	6.21	4.31	3.71	4.14	4.6	4.65	3.9	5.42	5.21	5.3
0.75	5.55	5.76	5.34	3.74	3.66	3.74	4.84	4.82	4.63	5.39	5.31	5.39
1.00	5.37	5.11	5.23	4.00	3.72	4.86	4.7	4.99	4.61	5.21	5.55	4.67
SEM	0.24	0.4	0.52	0.34	0.30	0.45	0.19	0.34	0.28	0.21	0.32	0.38

[1]Force (kg) necessary to penetrate cooked muscle.

istered with 0.5% limestone. In agreement with the current study, previous studies have indicated that troponin-T degradation increases with aging (Montgomery et al., 2000, 2002, 2004; Foote et al., 2004; Wertz et al., 2004). Though total degradation was very low in this study, treatment with 500 mg of 25-OH D_3 and 0.50% dietary limestone or 0 mg of 25-OH D_3 and 1.0% dietary limestone seemed to increase postmortem protein degradation in some muscles. In some cases, the effect of 25-OH D_3 seemed to be increased with increased dietary calcium, but in others, increased calcium decreased the effect of 25-OH D_3.

Texture Analysis

Star probe analysis was used to measure the tenderness of steaks from the adductor, gracilus, pectineus, sartorius, semimembranosus, vastus intermedius, and vastus lateralis muscles aged for 1, 3, or 7 d. Results for the main effects of 25-OH D_3 and dietary limestone for affected muscles are shown because no interactions existed (Table 7). The pectineus and the vastus intermedius muscles seemed to exhibit the largest response to treatments, but the differences were not significant ($P \geq 0.10$). The fact that none of these differences reached significance is likely an effect of the small sample size in this study because other studies (Montgomery et al., 2000, 2002; Karges et al., 2001) have found that supplementation with vitamin D_3 improves tenderness because of increased postmortem proteolysis. Also, given the age of the cows treated in the current study, the possibility of different mechanisms at work also must be considered (Huff and Parrish, 1993; Huff-Lonergan et al., 1995).

Results from the current experiment indicate that 500 mg of 25-OH D_3 administered as a single bolus 7 d before slaughter improves certain indicators of tenderness of steaks from the round of beef cows, but improved tenderness was not observed. The 25-OH D_3 is as effective as vitamin D_3 at increasing plasma calcium and has the potential to improve tenderness of beef without leaving high concentrations of vitamin D_3 metabolites in muscle. In fact, the concentrations of 25-OH D_3 found in beef from treated cows even could be beneficial to consumers. Withdrawing and replenishing limestone from the diet does not seem to increase calcium in the blood above the normal biological concentration of approximately 9 mg/dL, so that treatment is not likely to increase calpain activity in muscle or result in improved tenderness.

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